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Activation of Cap-Dependent Translation by Mucosal Human Papillomavirus E6 Proteins Is Dependent on the Integrity of the LXXLL Binding Motif

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The human papillomavirus (HPV) type 16 (HPV16) E6 protein can stimulate mechanistic target of rapamycin complex 1 (mTORC1) signaling and cap-dependent translation through activation of the PDK1 and mTORC2 kinases. Here we report that HPV18 E6 also enhances cap-dependent translation. The integrity of LXXLL and PDZ protein binding domains is important for activation of cap-dependent translation by high-risk mucosal HPV E6 proteins. Consistent with this model, low-risk mucosal HPV6b and HPV11 E6 proteins, which do not contain a PDZ protein binding motif, also activate cap-dependent translation and mTORC1, albeit at a lower efficiency than high-risk HPV E6 proteins. In contrast, cutaneous HPV5 and HPV8 E6 proteins, which lack LXXLL and PDZ motif protein binding, do not enhance cap-dependent translation. Mutagenic analyses of low-risk HPV E6 proteins revealed that association with the LXXLL motif containing ubiquitin ligase E6AP (UBE3A) correlates with activation of cap-dependent translation. Hence, activation of mTORC1 and cap-dependent translation may be important for the viral life cycle in specific epithelial tissue types and contribute to cellular transformation in cooperation with other biological activities of high-risk HPV E6-containing proteins.

Human papillomaviruses (HPVs) are small double-stranded DNA viruses with a tropism for mucosal and cutaneous epithelial cells. Over 200 HPV types have been identified, of which approximately 30 infect mucosal epithelia. Mucosal HPV types are further categorized by their propensity to cause lesions that can progress to carcinogenesis. Low-risk mucosal HPV types, including HPV type 6b (HPV6b) and HPV11, are most frequently associated with benign genital warts, whereas high-risk mucosal HPV types, such as HPV16 and HPV18, cause squamous intraepithelial lesions that can progress to cancer. High-risk HPVs are associated with over 99% of cervical cancers and at a lower prevalence with other anogenital cancers. High-risk HPV infections also account for approximately 25% of all oral cancers, particularly those of the oropharynx and tonsil (reviewed in references 15 and 23). HPV-induced cervical carcinogenesis is often associated with viral genome integration into host chromosomal DNA, causing dysregulated HPV E6 and E7 expression. The high-risk HPV E6 and E7 proteins are sufficient for the induction and maintenance of transformation of cervical epithelial cells in culture and cause cancers in transgenic mouse models (reviewed in reference 15). These proteins lack intrinsic enzymatic and nucleic acid binding activities and, therefore, modulate cellular processes through the association with and functional modification of cellular protein complexes.

The best-characterized cellular targets of high-risk HPV E6 and E7 proteins are the p53 and retinoblastoma (pRB) tumor suppressors, respectively (reviewed in references 7 and 16). High-risk HPV E6 proteins form a tripartite complex with p53 and the cellular ubiquitin ligase E6AP (UBE3A), targeting p53 for ubiquitination and proteasome-mediated degradation (22). High-risk HPV E6 oncoproteins also associate with cellular PDZ proteins, including MUPP1 (MPDZ), human disc large (DLG1), human scribble (SCRIB), MAGI1, PTPN13, and PTPN3, through the HPV E6 carboxyl-terminal PDZ binding domain. Associated cellular PDZ proteins may also be targeted for proteasomal degradation via the E6/E6AP complex (reviewed in reference 27). High-risk HPV E6 proteins also contribute to cellular transformation and immortalization through transcriptionally activating TERT, the catalytic protein subunit of human telomerase (8). Additional potential cellular targets of HPV E6 proteins have also been reported (9).

Despite differences in the carcinogenic potential of the lesions that they cause, low-risk and high-risk HPV E6 proteins both associate with proteins that contain LXXLL motifs. LXXLL motifs are defined as leucine-rich amphipathic helices with limited leucine substitution for hydrophobic residues and at least one negatively charged amino acid in an X position (reviewed in reference 7). Low- and high-risk HPV E6 proteins have been shown to associate with the E6AP ubiquitin ligase via its LXXLL motif (1). Low-risk HPV E6/E6AP complexes, however, do not bind and ubiquitinate p53, and only very few substrates have been proposed. The proapoptotic BAK1 protein associates with high- and low-risk mucosal HPV E6 proteins and may be a substrate for the HPV E6/E6AP complex (26). Cutaneous HPV E6 proteins have also been shown to associate with and target BAK1 for degradation, despite their lack of an LXXLL binding motif and thus the inability to form a stable complex with E6AP (29). A two hybrid screen in *Saccharomyces cerevisiae* using HPV18 and HPV6 E6 as the bait identified the G protein and mitogen-activated protein kinase (MAPK) suppressor GPS2 as a putative binding partner of high- and low-risk HPV E6 proteins. Ectopic expression of high-

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and low-risk HPV E6 proteins reduced GPS2 detection, suggesting that it may be targeted for degradation (3). Mucosal HPV E6 proteins have been reported to associate with LXXLL proteins other than E6AP, including paxillin and E6BP (RCN2) (4, 28). Paxillin and E6BP were shown to associate with the bovine papillomavirus 1 (BPV1) and high-risk HPV16 E6 proteins but not with low-risk HPV6b or HPV11 E6 proteins, despite the conserved LXXLL protein binding motif (2, 28, 30).

The mechanistic target of rapamycin complex 1 (mTORC1) signaling pathway is a key regulator of cellular metabolism. mTORC1 responds to a variety of cellular signals, including, but not limited to, nutrient and growth factor availability and cellular ATP and amino acid levels. These signals converge upon the mTORC1 kinase complex, which consequently regulates downstream cellular processes, including cell proliferation, growth, and size. Mechanistically, mTORC1-mediated regulation of cellular anabolic processes is at least in part through activation of cap-dependent translation. mTORC1 regulates this process by phosphorylating the mitogen-activated p70S6 kinase (S6K), which in turn phosphorylates and activates the ribosomal protein S6, which is involved in translation initiation. mTORC1 also phosphorylates the eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1). 4E-BP1 hyperphosphorylation relieves repression of the translation initiation factor eIF4E to stimulate translation (reviewed in reference 13).

Immunohistochemistry analyses of S6K and mTORC1 phosphorylation showed evidence for mTORC1 hyperactivity in cervical carcinomas and high-grade squamous intraepithelial lesions (5, 20). It has been reported that the HPV16 E6 protein activates mTORC1 signaling through E6AP-dependent degradation of the mTORC1 inhibitor tuberous sclerosis 2 (TSC2). TSC2 degradation was restricted to HPV16 E6 (12). We have previously reported that HPV16 E6 activates mTORC1 signaling and cap-dependent translation through activation of two upstream kinases, PDK1 and mTORC2, and detected no evidence for TSC2 degradation in HPV16 E6-expressing cells (25).

Here we report that the integrity of LXXLL motif and PDZ protein binding domains is important for high-risk HPV E6 proteins to activate cap-dependent translation. Low-risk HPV6b and HPV11 E6 proteins that do not contain a PDZ protein binding motif activate cap-dependent translation and mTORC1 at a reduced level. In contrast, cutaneous beta HPV5, and HPV8 E6 proteins, which do not associate with LXXLL motif and PDZ proteins, do not enhance cap-dependent translation. Association of low-risk HPV E6 proteins with the LXXLL motif containing ubiquitin ligase E6AP (UBE3A) correlates with activation of cap-dependent translation.

MATERIALS AND METHODS

Plasmids. Plasmids used in this study included a set of human β-actin promoter-driven expression vectors, p1318 (control), p1436no* (HPV16 E6), HPV18 E6, HPV6b E6, HPV11 E6, HPV5 E6, and HPV8 E6 (18); a set of pCMV BamNeo vectors with Flag-hemagglutinin epitopes fused to the amino termini of HPV E6 protein, pNCMV, pNCMV HPV16 E6no*, pNCMV HPV18 E6no*, pNCMV HPV6b E6, and pNCMV HPV11 E6; and a set of lentiviral vectors, pLentiN (control), pLenti HPV16 NE6no*, pLenti HPV18 NE6no*, pLenti HPV6b NE6, pLenti HPV11 NE6, pLenti HPV5 NE6, and pLenti HPV8 NE6 and were generated by Gateway cloning into the pLenti6.3/V5 TOPO Gateway-compatible vector (Invitrogen). HPV E6 mutants were generated by directed mutagenesis (QuikChange; Stratagene). The HPV16 and HPV18 E6 expression vectors were mutated such that they do not splice to form the E6* or E6** species (24). The resulting HPV E6 mutants are referred to as E6no*. E6no* mutants carry a coding mutation, V42L and V44L in HPV16 and HPV18 E6no*, respectively, which does not interfere with the ability of HPV16 E6 to contribute to epithelial cell immortalization (24). E6AP binding-defective HPV6b, -11, and -18 E6 mutants were generated in the pNCMV, β-actin, and pLentiN6.3 backgrounds on the basis of data that implicated HPV16 residues L110, I128, and G130 in efficient E6AP binding (11). A PDZ protein binding-defective HPV16 E6 mutant with a truncation of the six carboxyl-terminal residues was made in the β-actin and pLentiN6.3 backgrounds. The pFR_CrPV_xb bicistronic firefly/*Renilla* luciferase vector was used for luciferase reporter assays and was obtained from Phil Sharp through Addgene (plasmid 11509) (21).

Cell lines and culture. 293T and U2OS cells (ATCC) were maintained in Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin. Primary human foreskin keratinocytes (HFKs) were isolated from anonymous newborn circumcisions as previously described (14) and maintained in keratinocyte serum-free medium (KSFM) supplemented with human recombinant epidermal growth factor 1-53, bovine pituitary extract (Invitrogen), 50 U/ml penicillin, 50 μg/ml streptomycin, 20 μg/ml gentamicin, and 1 μg/ml amphotericin B. HPV E6-expressing HFKs were generated by lentiviral infection with the corresponding pLenti6.3N vectors followed by blasticidin selection (3 μg/ml). All experiments were performed with HFKs passaged less than 10 times.

Western blotting and antibodies. Unless indicated otherwise, cell lysates were prepared using ML buffer (300 mM NaCl, 0.5% Nonidet P-40 [NP-40], 20 mM Tris-HCl [pH 8.0], 1 mM EDTA) supplemented with one complete EDTA-free protease inhibitor cocktail tablet (Roche) per 25 ml lysis buffer and one PhosSTOP phosphatase inhibitor cocktail tablet (Roche) per 7.5 ml lysis buffer (14). Lysates intended for hemagglutination (HA) immunoprecipitations were prepared using MC lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.5% NP-40) supplemented with one complete EDTA phosphatase inhibitor cocktail tablet (Roche). Lysates were cleared by centrifugation at 16,110 × *g* for 10 min at 4°C. Protein concentrations were determined using the Bradford method (Bio-Rad). Proteins were separated by SDS-PAGE and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). Unless otherwise noted, membranes were blocked in 5% nonfat dry milk in TBST (137 mM NaCl, 2.7 mM KCl, 25 mM Tris [pH 7.4], 0.1% Tween 20) and probed with the appropriate antibody. The following antibodies were used at a 1:1,000 dilution unless otherwise specified: β-actin (1501; Chemicon), p53 (Ab-6; Calbiochem), Flag (4 μg/ml; F3165; Sigma), UBE3A/E6AP (1:500; H00007337-M01; Novus Biologicals), and S6K (9202) and S6K T389 (9206) from Cell Signaling Technology. Secondary antimouse and antirabbit antibodies conjugated to horseradish peroxidase were used at dilutions of 1:10,000 and 1:15,000, respectively. Proteins were visualized by enhanced chemiluminescence (Perkin Elmer, Millipore) and exposed on Kodak BioMax XAR film or electronically acquired and quantified with a Kodak Image Station 4000R equipped with Kodak imaging software, version 4.0, or with a Carestream Gel Logic 4000 imager.

Immunoprecipitations. For HA immunoprecipitations, one 15-cm plate of 293T cells was seeded and $CaCl_2$ transfected with the appropriate pNCMV-based vector (empty vector, 16E6no*, 16E6no* I128T, 18E6no*, 18E6no* I130T, 11E6, 11E6 L111Q, 11E6 I127T, or 11E6 L129T). At 72 h posttransfection, cells were lysed and lysates were cleared with low-binding Durapore PVDF 0.45-μm-pore-size membrane spin filters (Millipore). Prewashed HA antibody-agarose conjugate (Sigma) was incubated with lysate for 2 h and washed, and sample buffer was added.

Transfections and luciferase assays. U2OS and primary HFKs were transfected and processed for luciferase assays (dual luciferase reporter kit; Promega) as described previously (25). The fold change in activity was determined by calculating the ratio of firefly luciferase activity to *Renilla* luciferase activity and comparing it to the ratio for control vector-trans-

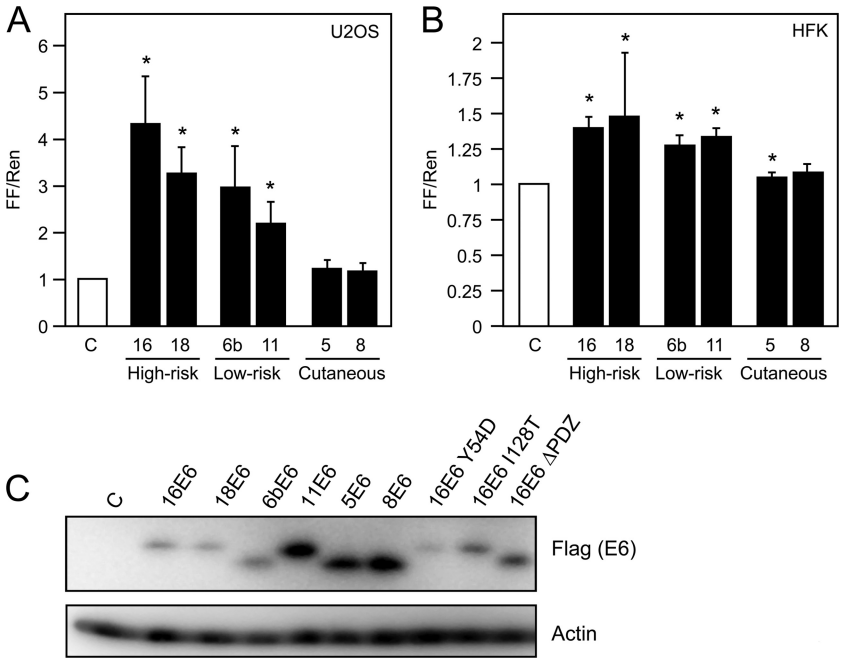

FIG 1 Mucosal but not cutaneous HPV E6 proteins increase cap-dependent translation. (A) U2OS cells were transiently cotransfected with the pFR_CrPV_xb bicistronic reporter construct and cytomegalovirus promoter-driven expression vectors for high-risk mucosal HPV E6 proteins (HPV16, HPV18), low-risk mucosal HPV E6 proteins (HPV6b, HPV11), cutaneous HPV E6 proteins (HPV5, HPV8), or empty vector (C) as a control. (B) Primary HFKs were transiently cotransfected with the pFR_CrPV_xb construct and human β-actin promoter-driven E6 expression vectors. Cells were lysed, and *Renilla* and firefly luciferase activities were measured at 48 h posttransfection. Firefly and *Renilla* luciferase values were normalized to the values for control vector-transfected cells and are presented as the fold change of normalized firefly luciferase relative to normalized *Renilla* luciferase (FF/Ren). The bar graphs depict averages and standard deviations of four independent experiments for U2OS and five independent experiments for HFKs. *, statistical significance ($P \leq 0.01$ and $P < 0.05$ for U2OS and HFKs, respectively). (C) Immunoblot analysis of various HPV E6 proteins after transient transfection of U2OS cells with lentiviral expression plasmids. An actin blot is shown as a loading control.

fected cells. At least three independent experiments were performed, and the Student *t* test was used to calculate statistical significance.

RESULTS

Mucosal but not cutaneous HPV E6 proteins enhance cap-dependent translation. Our previous studies showed that HPV16 E6 increases cap-dependent translation (25). Based on these findings, we evaluated the ability of E6 proteins encoded by other HPV types to activate cap-dependent translation in U2OS osteosarcoma cells and primary HFKs. U2OS cells were selected for these experiments because of their high transfection efficiency and because they support the full HPV life cycle (6), and HFKs, while more cumbersome, represent a physiologically relevant cell type. We utilized a bicistronic luciferase reporter plasmid, pFR_CrPV_xb, expressing firefly and *Renilla* luciferase as a single transcript from the minimal thymidine kinase promoter (21). Firefly luciferase is translated by a cap-dependent mechanism, whereas *Renilla* luciferase is translated by an internal ribosome entry site (IRES)-dependent, cap-independent mechanism (21). Transient coexpression of high-risk mucosal HPV16 and HPV18 E6 proteins robustly activated cap-dependent translation in U2OS cells (4.32 ± 1.04-fold, $P < 0.001$, and 3.24 ± 0.58-fold, $P < 0.001$, respectively). Low-risk mucosal HPV6b and HPV11 E6 proteins also activated cap-dependent translation, albeit not quite as efficiently as high-risk HPV E6 proteins (2.94 ± 0.91-fold, $P < 0.001$, and 2.19 ± 0.47-fold, $P < 0.003$, respectively), whereas cotransfection of the cutaneous HPV5 and HPV8 E6 proteins had no significant effect (1.21 ± 0.18-fold, $P = 0.059$, and 1.16 ± 0.19-fold, $P = 0.16$, respectively) (Fig. 1A). Luciferase reporter assays performed in primary HFKs yielded similar results, although, presumably due to the lower transfection efficiency of HFKs, the effects were not as pronounced as those in U2OS cells (Fig. 1B). Immunoblot analyses revealed that HPV16, HPV18, and HPV6b E6 proteins are expressed at similar levels, whereas HPV11, HPV5, and HPV8 E6 proteins are expressed at higher levels (Fig. 1C), suggesting that the differences in activation of cap-dependent translation do not simply reflect intrinsic differences in protein stability.

Hence, high- and low-risk mucosal but not cutaneous HPV E6 proteins can activate cap-dependent translation.

The LXXLL and PDZ binding motifs contribute to HPV16 E6 activation of cap-dependent translation. To evaluate sequences in HPV16 E6 that contribute to enhancing cap-dependent translation, we tested two HPV16 E6 mutants, Y54D and I128T, that disrupt the LXXLL binding motif and are therefore defective for E6AP binding (11) as well as an HPV16 E6 mutant with a truncation of the PDZ binding domain (ΔPDZ) in bicistronic luciferase reporter assays in primary HFKs. Compared to wild-type HPV16 E6, the HPV16 E6 Y54D and I128T LXXLL binding motif-defective mutants activated cap-dependent translation at a significantly reduced level (wild-type HPV16 E6, 1.80 ± 0.08-fold, and HPV16 E6 Y54D, 1.26 ± 0.23-fold, $P = 0.125$ relative to control and $P = 0.0177$ relative to HPV16 E6; HPV16 E6 I128T, 1.16 ± 0.11-fold, $P = 0.0873$ relative to control and $P = 0.0013$ relative to HPV16 E6) (Fig. 2). The HPV16 E6 ΔPDZ mutant was also significantly

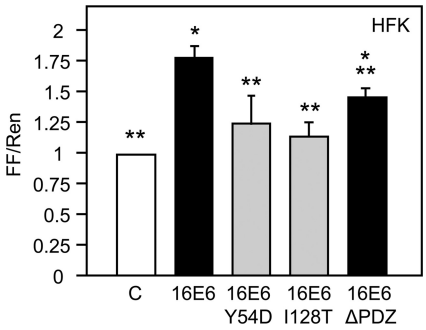

FIG 2 The LXXLL and PDZ binding motifs are important for the HPV16 E6-mediated increase in cap-dependent translation. (A) HFKs were transiently cotransfected with the pFR_CrPV_xb reporter construct and human β-actin promoter-driven expression vectors for wild-type HPV16 E6 (16E6), HPV16E6 Y54D (16E6 Y54D) and HPV16 E6 I128T (16E6 I128T) E6AP binding-defective mutants, HPV16E6 ΔPDZ (16 E6 ΔPDZ) PDZ binding-defective mutant, or empty vector (C) as a control. Cells were lysed, and firefly and *Renilla* luciferase activities were measured at 48 h posttransfection. Firefly and *Renilla* luciferase values were normalized to the values for control vector-transfected cells and are presented as the fold change of normalized firefly luciferase relative to normalized *Renilla* luciferase (FF/Ren). The bar graph depicts averages and standard deviations of three independent experiments *, statistical significance relative to empty vector ($P \leq 0.001$); **, statistical significance relative to wild-type HPV16 E6 ($P \leq 0.01$).

reduced but activated cap-dependent translation significantly above vector control levels (1.47 ± 0.07-fold, $P = 0.003$ relative to control and $P = 0.0066$ relative to HPV16 E6) (Fig. 2). Western blot analyses revealed that while the HPV16 E6 Y54D mutant was expressed at a lower level, the HPV16 E6 I128T and ΔPDZ mutants were expressed at similar or higher levels than wild-type HPV16 E6 (Fig. 1C), suggesting that the observed differences in stimulating cap-dependent translation do not simply reflect intrinsic differences in protein expression.

Hence, these results are consistent with the model that the ability to associate with LXXLL motif-containing cellular proteins such as E6AP as well as association with PDZ proteins contributes to the ability of HPV16 E6 to activate cap-dependent translation.

The LXXLL binding motif of low-risk mucosal HPV E6 proteins is critical for activation of cap-dependent translation. Low-risk HPV E6 proteins activate cap-dependent translation less efficiently than high-risk HPV E6 proteins. Low-risk HPVs lack a carboxyl-terminal PDZ binding site, and while they share an LXXLL binding motif and associate with E6AP, they do not efficiently interact with p53. To determine whether the integrity of the LXXLL binding motif is necessary for the ability of low-risk HPV E6 proteins to activate cap-dependent translation, we generated three mutations, L111Q, I127T, and L129T, within the HPV6b and HPV11 E6 sequences. Previous studies identified leucine 111 in HPV11 E6 to be important for association with E6AP through its LXXLL motif (1, 11). The mutation of isoleucine 127 and leucine 129 is based upon the requirement of homologous amino acids in HPV16 E6 to associate with the LXXLL protein E6AP (Fig. 3A) (11).

We first assessed disruption of the LXXLL binding motif by evaluating E6AP association by transient transfection of 293T cells followed by HA immunoprecipitation and E6AP Western blotting. Wild-type and LXXLL binding motif/E6AP binding-defective HPV16 and HPV18 E6 proteins were used as a control. These experiments confirmed that HPV16 E6 binds E6AP, whereas the HPV16 E6 I128T mutant is defective for E6AP binding (Fig. 3B, left). Similar results were obtained for HPV18 E6 and the HPV18 I130T LXXLL binding motif mutant (Fig. 3B, middle). Consistent with previously published results (1), the HPV6b and HPV11 E6 L111Q mutants are defective for E6AP association. The HPV6b and HPV11 E6 I127T mutants exhibit reduced E6AP binding, whereas the HPV6b and HPV11 E6 L129T mutants retained some E6AP binding (Fig. 3B, middle and right).

We used this set of mutants to determine whether association with LXXLL proteins such as E6AP correlates with increased cap-dependent translation by low-risk HPV E6 proteins. U2OS cells were transiently cotransfected with high- and low-risk HPV E6 proteins, their respective LXXLL binding motif mutants, and the bicistronic luciferase reporter. Similar to what we observed with HPV16 E6, transfection of the LXXLL binding motif/E6AP binding-defective HPV18 E6 I130T mutant caused a significant decrease in activation of cap-dependent translation (0.91 ± 0.24-fold compared to 1.67 ± 0.17-fold for wild-type HPV18 E6, $P = 0.006$ relative to wild-type HPV18 E6). Consistent with previous experiments (Fig. 1), transfection of low-risk HPV6b and HPV11 E6 caused a significant (1.33 ± 0.06-fold, $P < 0.001$, and 1.20 ± 0.10-fold, $P < 0.001$; P values compared to vector control) albeit less dramatic increase in cap-dependent translation compared to that for high-risk HPV E6. The integrity of the LXXLL binding motif is also important for low-risk HPV E6 proteins to activate cap-dependent translation, as it is significantly reduced upon expression of the E6AP association-defective HPV6b E6 L111Q and I127T mutants (HPV6b E6 L111Q, 1.05 ± 0.14-fold, $P = 0.001$; HPV6b E6 I127T, 1.06 ± 0.09-fold, $P < 0.001$; P values relative to wild-type HPV6b E6). The HPV6b L129T mutant, which retained E6AP binding activity, despite being expressed at lower levels than wild-type HPV16 E6, enhanced cap-dependent translation to a level similar to that for wild-type HPV6b E6 (1.35 ± 0.16-fold, $P = 0.634$ relative to wild-type HPV6b E6). Similarly, the E6AP binding-defective HPV11 E6 L111Q and I127T mutants caused reduced cap-dependent translation (HPV11 E6 L111Q, 1.03 ± 0.18-fold, $P = 0.083$; HPV11 E6 I127T, 1.02 ± 0.12-fold, $P = 0.022$; P values relative to wild-type HPV11 E6), but the HPV11E6 L129T mutant, which retained some E6AP binding, also consistently reduced cap-dependent translation (1.04 ± 0.09-fold, $P = 0.019$) (Fig. 3C).

Hence, these experiments are consistent with the model that the integrity of the LXXLL binding motif as measured by E6AP binding contributes to activation of cap-dependent translation by high-risk as well as low-risk mucosal HPV E6 proteins.

The LXXLL binding motif contributes to the ability of mucosal HPV E6 proteins to activate mTORC1 signaling. We next tested the previously characterized LXXLL binding motif/E6AP binding-defective HPV16 E6 Y54D and I128T mutants (11), as well as the PDZ protein binding-defective HPV16 E6 ΔPDZ mutant, for mTORC1 activation, using S6K phosphorylation as a surrogate marker. HFKs with stable expression of these HPV16 E6 mutants were generated. Cells with expression of the LXXLL binding motif/E6AP binding-defective HPV16 E6 I128T and Y54D mutants exhibited S6K phosphorylation dramatically reduced from that by wild-type HPV16 E6-expressing cells (Fig. 4A). Cells with expression of the HPV16 E6 ΔPDZ mutant, however, showed S6K phosphorylation similar to that of wild-type HPV16 E6-expressing cells (Fig. 4A). These results are similar to what we observed with the bicistronic luciferase reporter assays, in that the

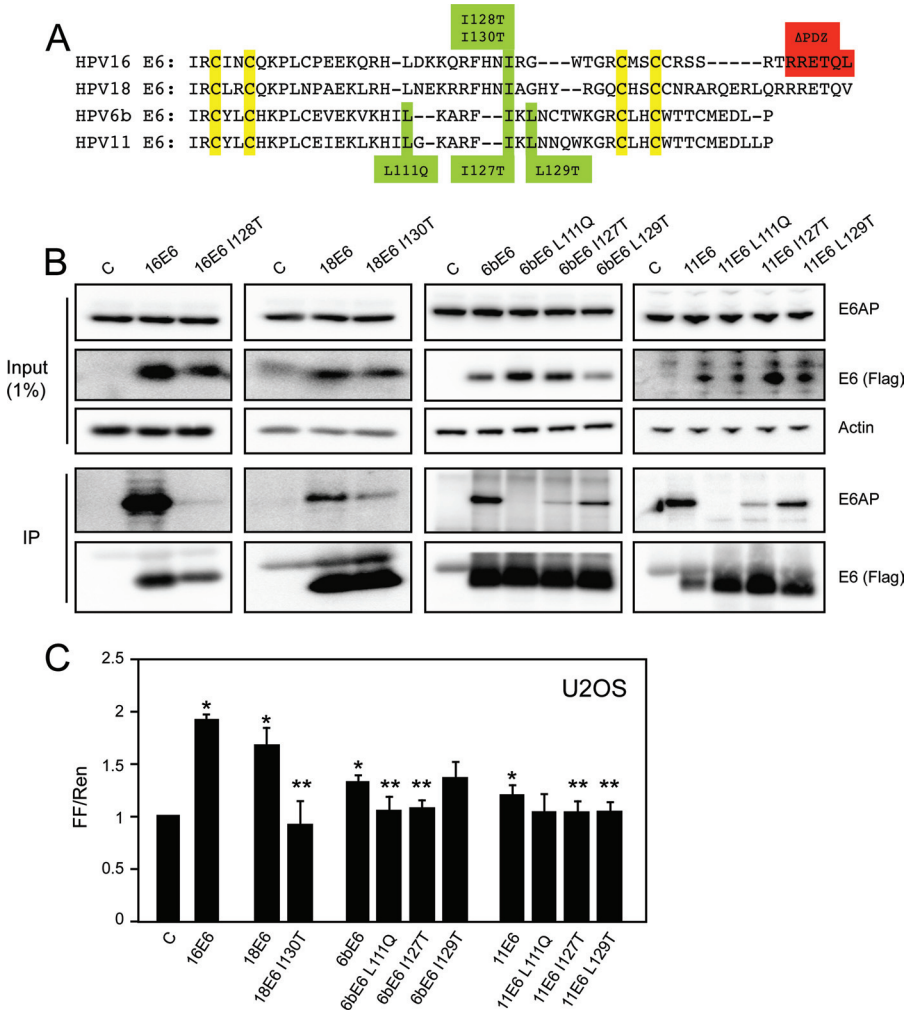

FIG 3 High- and low-risk HPV E6 proteins increase cap-dependent translation through overlapping mechanisms. (A) Schematic representation of the HPV E6 mutants used in these experiments. The amino acid sequences of the carboxyl-terminal zinc binding domains of HPV16, HPV18, HPV6b, and HPV11 are shown with the positions of the cysteine residues highlighted in yellow. The positions of the various LXXLL protein binding motif mutants are highlighted in green, and the amino acid residues deleted in the HPV16 E6 ΔPDZ mutant are highlighted in red. (B) Western blot analysis of HA immunoprecipitations (IP) from 293T cells transiently transfected with N-terminally Flag-HA-tagged cytomegalovirus (NCMV) promoter-driven E6 expression vectors. (Upper three panels) One percent of input for immunoprecipitations; (lower two panels) E6AP coprecipitated by the various HPV E6 proteins (detected by Flag epitope-specific antibody). (C) Analysis of cap-dependent translation. U2OS cells were transiently cotransfected with the pFR_CrPV_xb bicistronic reporter construct and the human β-actin promoter-driven E6 expression vectors or empty vector as a control. Cells were lysed, and *Renilla* and firefly luciferase activities were measured at 48 h posttransfection. Firefly and *Renilla* luciferase values were normalized to those for control vector-transfected cells and are presented as the fold change of normalized firefly luciferase relative to normalized *Renilla* luciferase (FF/Ren). The bar graph depicts averages and standard deviations from seven independent experiments, *, statistical significance relative to empty vector ($P < 0.001$); **, statistical significance relative to the corresponding wild-type HPV E6 protein ($P \leq 0.022$).

E6AP binding-defective HPV16 E6 mutants showed a more profound defect in activating cap-dependent translation than the ΔPDZ mutant (Fig. 2).

Given that the ability to associate with LXXLL motif-containing proteins such as E6AP is conserved with low-risk and high-risk HPV E6 proteins, we next investigated whether the low-risk HPV11 E6 protein shares the ability to activate mTORC1 signaling and if so whether the integrity of the LXXLL motif binding sequence is also important. We consistently detected a very minor increase in S6K phosphorylation in the HPV11 E6-expressing HFKs. HFKs with expression of the E6AP binding-defective HPV11 L111 and I127 E6 mutants consistently showed lower levels of S6K phosphorylation than wild-type HPV11 E6-expressing cells (Fig. 4B).

Hence, the integrity of the LXXLL motif is important for activation of mTORC1 signaling by HPV16 E6 and may also contribute to mTORC1 activation by low-risk HPV E6 proteins.

DISCUSSION

We previously reported that HPV16 E6 activates mTORC1 signaling through activation of PDK1 and mTORC2 (25). We also showed that HPV16 E6 increases cap-dependent translation and that this is at least in part dependent on mTORC1 activation (25).

Here we report that the ability to activate mTORC1 and increase cap-dependent translation is conserved among high- and low-risk mucosal HPV E6 proteins (Fig. 1) but that the low-risk mucosal HPV6b and 11 E6 proteins do so less efficiently. While HPV6b E6 is expressed at low levels, HPV11 E6 is expressed at

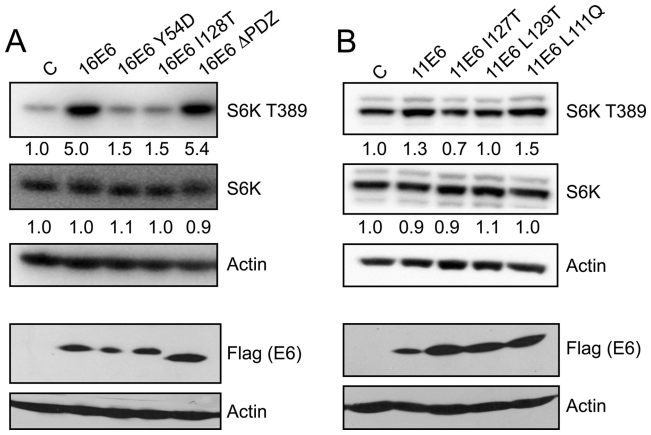

FIG 4 The LXXLL binding motif is important for mTORC1 activation by mucosal HPV E6 proteins. (A) Western blot analysis of S6K T389 phosphorylation in HFKs with pLentiN6.3-based lentiviral vectors expressing wild-type HPV16 E6 (16E6), HPV16 E6 Y54D (16E6 Y54D) and HPV16 E6 I128T (16E6 I128T) E6AP binding-defective mutants, HPV16 E6 ΔPDZ (16E6 ΔPDZ) PDZ binding-defective mutant, or empty vector (C). Blots for total S6K and actin are also shown. Quantifications relative to actin are indicated below each panel. Expression of the corresponding Flag epitope-tagged E6 proteins in this HFK population is also shown. (B) Western blot analysis of S6K T389 phosphorylation in HFKs with pLentiN6.3-based vectors expressing wild-type HPV11 E6, the E6AP binding-defective HPV11 E6 L111Q (11E6 L111Q), HPV11 E6 I127T (11E6 I127T), or HPV11 E6 I129T (11E6 I129T) mutants, or empty vector (C). Blots for total S6K and actin are also shown. Quantifications relative to actin are indicated below each panel. Expression of the corresponding Flag epitope-tagged E6 proteins in this HFK population is also shown.

levels that are higher than HPV16 E6, and hence, the observed defect of low-risk HPV E6 proteins in activation of cap-dependent translation is not strictly correlated to levels of expression (Fig. 1C). In contrast, cutaneous HPV E6 proteins do not activate cap-dependent translation, despite the fact that they are expressed at much higher levels than HPV16 E6.

Our results show that LXXLL motif and PDZ protein binding are each required for enhancement of cap-dependent translation by HPV16 E6 (Fig. 2 and 3C). Consistent with this model, low-risk HPV6b and HPV11 E6 proteins, which lack a PDZ binding motif but can associate with LXXLL motif proteins such as E6AP, activate cap-dependent translation at reduced levels (Fig. 1 and 3C). Cutaneous HPV5 and HPV8 E6 proteins, which lack PDZ as well as LXXLL motif protein (E6AP) binding, do not activate cap-dependent translation (Fig. 1). We further analyzed the importance of the association with LXXLL motif proteins by generating HPV6b and HPV11 E6 LXXLL motif protein binding mutants and assessed E6AP association (Fig. 3B). These experiments revealed that LXXLL motif protein binding is also required for the low-risk mucosal HPV6b and HPV11 E6 proteins to activate cap-dependent translation (Fig. 3) and potentially mTORC1 activation (Fig. 4B). Considering that the E3 ubiquitin ligase E6AP is the only LXXLL motif partner shared by low-risk and high-risk mucosal HPV E6 proteins, it is an obvious candidate to mediate enhanced cap-dependent translation and mTORC1 activation. Since E6 association with E6AP does not inactivate E6AP ubiquitin ligase activity but rather retargets it to E6-associated cellular proteins, the potential contribution of E6AP cannot be addressed by a simple RNA interference depletion experiment. Given that low-risk HPV E6 proteins do not cause E6AP-dependent p53 ubiquitination and degradation, it is unlikely that E6/E6AP-mediated p53 inactivation significantly contributes to activation of mTORC1 and cap-dependent translation. The only E6/E6AP substrate that was reported to be shared between low-risk and high-risk mucosal HPV E6 proteins is the proapoptotic BCL2 family member BAK1, and it is difficult to envision how BAK1 degradation might cause increased cap-dependent translation. Moreover, cutaneous HPV E6 proteins also target BAK for degradation, yet they do not activate cap-dependent translation.

It is possible, however, that E6AP binding is required for the HPV E6-mediated increase in mTORC1 and cap dependent, but the ubiquitin ligase activity of E6AP may be dispensable. It has been reported that E6AP may serve as a transcriptional coactivator of estrogen receptor alpha (ERα) and that the ubiquitin ligase activity is dispensable for this activity (19). Several ERα target genes have been implicated in upstream signaling events of mTORC1 and cap-dependent translation, including insulin growth factor binding protein 4 (IGFBP4), ERBB4, as well as other growth factor- and metabolism-associated genes such as NDRG1, a downstream effector of mTORC2 signaling (10).

Last but not least, it is possible that binding to a cellular LXXLL domain-containing protein other than E6AP is necessary for HPV E6-mediated activation of cap-dependent translation. Future experiments will be focused on directly addressing the potential mechanistic contributions of E6AP and/or other LXXLL motif-containing cellular proteins in activation of mTORC1 signaling and enhancement of cap-dependent translation by mucosal HPV E6 proteins.

The shared ability of high-risk and low-risk mucosal HPV E6 proteins to activate mTORC1 and enhance cap-dependent translation likely reflects a common requirement during the viral life cycle. Given that all HPVs, including those that infect the cutaneous epithelia, require adequate production of viral and cellular proteins necessary for viral genome replication and progeny virion production in terminally differentiated, presumably nutrient-deprived keratinocytes, it is surprising that cutaneous HPV E6 proteins do not increase cap-dependent translation. One might hypothesize that the specificity of mucosal HPV E6 activation of mTORC1 and cap-dependent translation suggests that mucosal HPV E6 proteins evolved a distinct repertoire of biological properties as a result of their different epithelial tropisms. Successful viral genome replication or progeny virion production in the mucosal epithelium involves unique requirements as a result of different gene expression profiles. Consistent with this notion, introduction of HPV16 and HPV5 long control region (LCR)-driven reporter constructs into cutaneous and mucosal epithelial cells demonstrated that appropriate cellular context is important for robust transcriptional activation (17). Given these apparent differences in the cellular milieus of cutaneous and mucosal epithelia, it is thus tempting to speculate that corresponding HPV types may have evolved distinct molecular strategies to optimally exploit the available host cellular environment.

In conclusion, the results presented here suggest that activation of mTORC1 and cap-dependent translation is shared between low-risk and high-risk mucosal but not with cutaneous HPV E6 proteins. Multiple biochemical activities of high-risk HPV E6 proteins, including binding to LXXLL motif- and PDZ motif-containing proteins, contribute to activation of cap-dependent translation. Given that the PDZ binding-deficient low-risk HPV6b and HPV11 E6 proteins retain some potential to activate cap-depen-

dent translation (Fig. 1), which is at least in part dependent on their ability to associate with cellular LXXLL domain proteins (Fig. 3B and C), we hypothesize that high-risk E6 proteins activate cap-dependent translation through pathways that are at least in part independent.

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